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Vonlanthen, Mireille ; Connelly, Colleen M ; Deiters, Alexander ; Linden, Anthony ; Finney, Nathaniel S

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# Thiourea-based fluorescent chemosensors for aqueous metal ion detection and cellular imaging

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and Nathaniel S. Finney<sup>a,b,\*</sup>

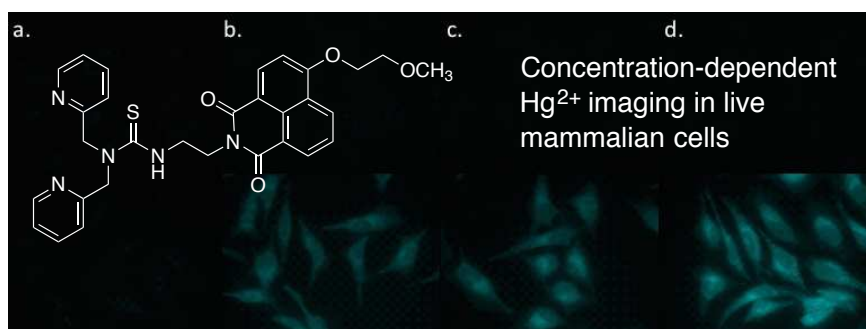
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## Abstract

We report three significant advances in the use of thioureas as reporting elements for metal-responsive fluorescent chemosensors. First, based on the crystal structure of a chemosensor analog, we provide a deeper understanding of the details of the thiourea coordination environment. Second, we describe a new generation of chemosensors with higher affinity for Zn<sup>2+</sup> and Cd<sup>2+</sup> than was

observed in earlier probes, expanding the scope of this type of probe beyond  $\text{Hg}^{2+}$  detection. Third, we show that a thiourea-based chemosensor can be employed for fluorescence microscopy imaging of  $\text{Hg}^{2+}$  ion concentrations in living mammalian cells.

## Introduction

As an analytical technique, fluorescence is remarkable for combining great sensitivity with ease of measurement. A central liability is that few analytes of interest are intrinsically fluorescent. This discrepancy has driven the development of fluorescent chemosensors, molecules which convert reversible association with a non-fluorescent analyte into a fluorescence response.<sup>1</sup> Fluorescent chemosensor development has been particularly effective for metallic species, and selective probes for several metal ions of biological and environmental relevance (e.g.  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Hg}^{2+}$ ) are now available.<sup>2-4</sup>

The dominant approach to turning reversible metal binding into a fluorescence response is the disruption of the electronic interaction between a nitrogen atom lone pair and a proximal fluorophore via metal coordination by nitrogen.<sup>1</sup> Most common are the use of benzylic amine-fluorophore conjugates, in which photoinduced electron transfer (PET) from nitrogen quenches fluorescence, and anilinic fluorophores, in which the nitrogen lone pair is engaged in the formation of an intramolecular charge transfer (ICT) excited state. In the former case,

protonation or coordination suppresses quenching and leads to enhanced emission. In the latter, protonation or coordination leads primarily to variation in the fluorophore emission wavelength, although concomitant intensity variation is not uncommon.

While these nitrogen-centric motifs have proven quite general,<sup>1</sup> reliance on nitrogen coordination for signaling possesses two characteristic shortcomings: false positive signaling resulting from protonation (i.e. pH sensitivity) and an exclusive reliance on nitrogen coordination chemistry for signaling.

In an effort to address these issues, we have worked to develop sulfur-based functional groups as reporting elements, and have recently reported the use of sulfoxides and thioureas to this end.<sup>5,6</sup> Building on our first generation of thiourea-naphthalimide conjugates,<sup>6</sup> we describe here a second generation of PET-based thiourea probes with high affinities for metal ions in aqueous media,<sup>7,8</sup> and show that they are both insensitive to excess acid and can be applied in cellular imaging.

## Results and Discussion

### *First Generation Thiourea Probes*

Our validation of thioureas as PET quenchers in metal-responsive fluorescent chemosensors has so far relied on naphthalimide chromophores because of their synthetic accessibility, chemical robustness and visible emission.<sup>9</sup> We have found that fluorescent chemosensors **MePic** and **DiPic** (Figure 1) have low

intrinsic quantum yields due to PET quenching of fluorescence emission by the thioureas (Figure 2).<sup>6</sup> Emission from the pendant naphthalimide reporter can be recovered by metal ion coordination of the thiourea (Table 1), with up to 20-fold fluorescence enhancement. The addition of a large excess of  $H^+$  (TFA) did not produce any change in the fluorescence emission of **MePic** or **DiPic**, validating our hypothesis that, in contrast to the amine-based probes described above, thioureas would provide  $H^+$ -independent signaling. Of the range of metal ions screened, the most significant fluorescence enhancements were observed for the  $d^{10}$  cations  $Zn^{2+}$ ,  $Cd^{2+}$  and  $Hg^{2+}$ , although **MePic** provides a modest response to  $Pb^{2+}$  as well. Of these,  $Hg^{2+}$  was by far the most tightly bound (Table 2), and the **DiPic**· $Hg^{2+}$  complex was found to form with  $K_d \leq 15$  nM in 9:1  $H_2O:CH_3CN$ .<sup>6,10</sup>

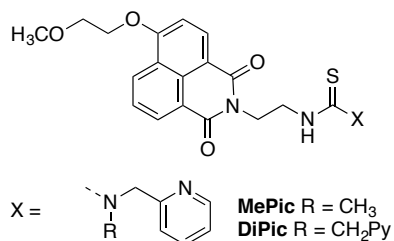


Figure 1. Fluorescent chemosensors **MePic** and **DiPic**. (Py = 2-pyridyl.)

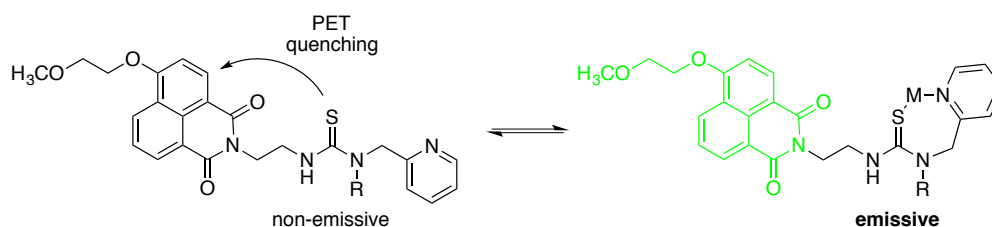


Figure 2. Fluorescent chemosensing with **MePic** and **DiPic**.

Table 1. Values of  $(I/I_0)_{\max}$  and  $\phi_{\max}$  for titrations of **MePic** and **DiPic**.<sup>a</sup>

	Zn <sup>2+</sup>	Cd <sup>2+</sup>	Hg <sup>2+</sup>	Pb <sup>2+</sup>
CH <sub>3</sub> OH <sup>b</sup>				
<b>MePic</b>	20.5 (0.62)	21.7 (0.65)	19.0 (0.57)	6.3 (0.19)
<b>DiPic</b>	13.0 (0.65)	5.0 (0.25)	7.4 (0.37)	— <sup>c</sup>
H <sub>2</sub> O <sup>d</sup>				
<b>MePic</b>	9.3 (0.28)	4.6 (0.14)	4.4 (0.13)	— <sup>c</sup>
<b>DiPic</b>	5.7 (0.29)	4.5 (0.23)	8.6 (0.43)	— <sup>c</sup>

<sup>a</sup>  $\phi_{\max}$  in parentheses.  $\phi_{\max} = \phi_{\text{initial}} \times (I/I_0)_{\max}$ .  $\phi_0(\text{MePic}) = 0.03$ ;  $\phi_0(\text{DiPic}) = 0.05$ .

<sup>b</sup> Titrations in CH<sub>3</sub>OH at 3.3  $\mu\text{M}$  chemosensor.

<sup>c</sup> No detectable fluorescence response.

<sup>d</sup> Titrations in 9:1 H<sub>2</sub>O:CH<sub>3</sub>OH at 3.3  $\mu\text{M}$  chemosensor.

Table 2. Apparent  $\log K_d$  (M) for titrations of **MePic** and **DiPic**.

	Zn <sup>2+</sup>	Cd <sup>2+</sup>	Hg <sup>2+</sup>	Pb <sup>2+</sup>
CH <sub>3</sub> OH <sup>a</sup>				
<b>MePic</b>	-3.2	-3.2	-5.8	-2.5
<b>DiPic</b>	-2.9	-3.1	-6.1	— <sup>b</sup>
H <sub>2</sub> O <sup>c</sup>				
<b>MePic</b>	-1.1	-2.1	-5.9	— <sup>b</sup>
<b>DiPic</b>	-0.9	-2.3	-6.0 <sup>d</sup>	— <sup>b</sup>

<sup>a</sup> Titrations in CH<sub>3</sub>OH at 3.3  $\mu\text{M}$  chemosensor.

<sup>b</sup> No detectable fluorescence response.

<sup>c</sup> Titrations in 9:1 H<sub>2</sub>O:CH<sub>3</sub>OH at 3.3  $\mu$ M chemosensor.

<sup>d</sup> Lower concentration titrations reveal that this log K<sub>d</sub> is at least -7.8. See ref. 6.

While the metal ion affinities of **MePic** and **DiPic** are similar, there is significant variation in maximum quantum yield, which reflects details of the thiourea coordination geometry that are not yet fully understood. We have obtained a crystal structure of the 1:1 complex of ZnCl<sub>2</sub> and a truncated analog of **DiPic** in which the fluorophore-bearing sidechain is replaced by a phenyl group (Figure 3).<sup>11</sup> The structure reveals a near-planar thiourea and coordination of the metal center by a single pyridine unit. Notably, the coordinated zinc atom is located significantly out of the thiourea plane, with an N-C-S-Zn dihedral angle of 57.40(14)°. Bis(thiourea) zinc chloride is known to have zinc coordinated in the plane of the thiourea,<sup>12</sup> consistent with  $\sigma$  coordination by an sp<sup>2</sup>-hybridized S atom – presumably the intrinsically preferred geometry. The observed deviation from in-plane coordination must be a consequence of geometric constraints imposed by the ring formation required for coordination of the picolyl group. From a functional point of view, these observations indicate that the coordination geometry responds strongly to structural details of the chelating group, which foreshadows the modulation of metal ion affinity and fluorescence response by ligand variation.

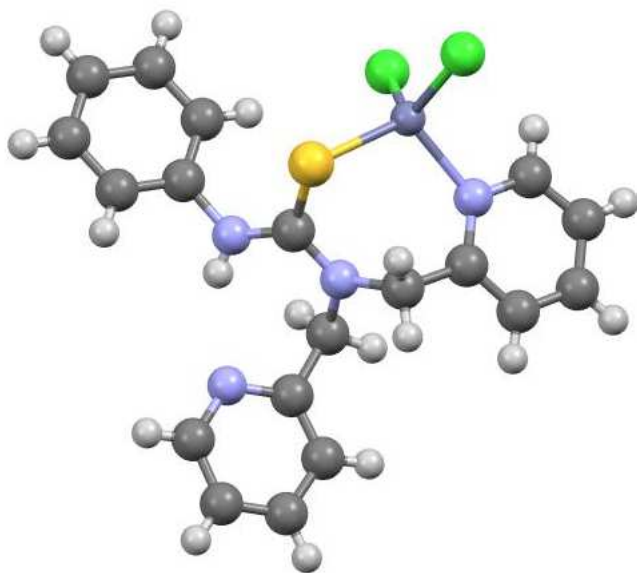


Figure 3. The molecular structure of the 1:1 complex between  $\text{ZnCl}_2$  and a truncated analog of **DiPic**.

Consistent with the crystal structure, all solution phase titrations of **MePic** and **DiPic** indicated 1:1 ligand:metal stoichiometry.<sup>6</sup> The  $\text{Hg}^{2+}$  affinity is sufficient to warrant evaluation of these probes in environmental samples, and it will be shown that **DiPic** is suitable for use in cellular imaging (vide infra).

A limitation of these first generation fluorescent chemosensors is that the nitrogen atoms of the thiourea remain conjugated to and coplanar with the thiocarbonyl. As a result, the thiourea nitrogen centers are not available for coordination, and only one of the distal *N*-substituents can participate in cooperative metal binding with the thiourea - as can be seen in the crystal structure (Figure 3). This accounts for the relatively low affinity observed for  $\text{Zn}^{2+}$  despite the presence of the high  $\text{Zn}^{2+}$ -affinity dipicolyl fragment in **DiPic**, and for



the similarities in metal affinity between **MePic** and **DiPic**. In conjunction with the induced deviation from optimal thiourea coordination discussed above, overcoming this limitation clearly requires more extended ligand domains with a greater degree of flexibility in order to accommodate alternate coordination geometries.

### *Second Generation Thiourea Probes*

In order to increase the flexibility and coordination denticity of the metal binding domains, analogs with ethylene diamine (En) spacers, **EnMePic** and **EnDiPic**, were prepared. In these molecules, the picolyl-bearing nitrogen is no longer conjugated to the thiocarbonyl, removing the associated geometric constraint and freeing the lone pair for binding (Figure 4; Scheme 1). Mono-Boc-protected ethylenediamine was either dialkylated to form the dipicolyl derivative (**1**) or monoalkylated by reductive amination (**3**).<sup>13</sup> In the latter case, *N*-methylation provided the requisite protected diamine.<sup>14</sup> Following removal of the Boc group, the free amines (RNH<sub>2</sub>; **2**, **4**) were reacted with a common imidazolyl thiourea (**5**), described previously,<sup>6</sup> to afford **EnMePic** or **EnDiPic** in modest yield.<sup>15</sup>

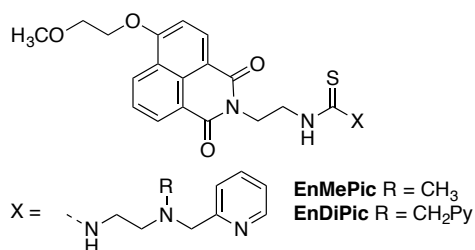
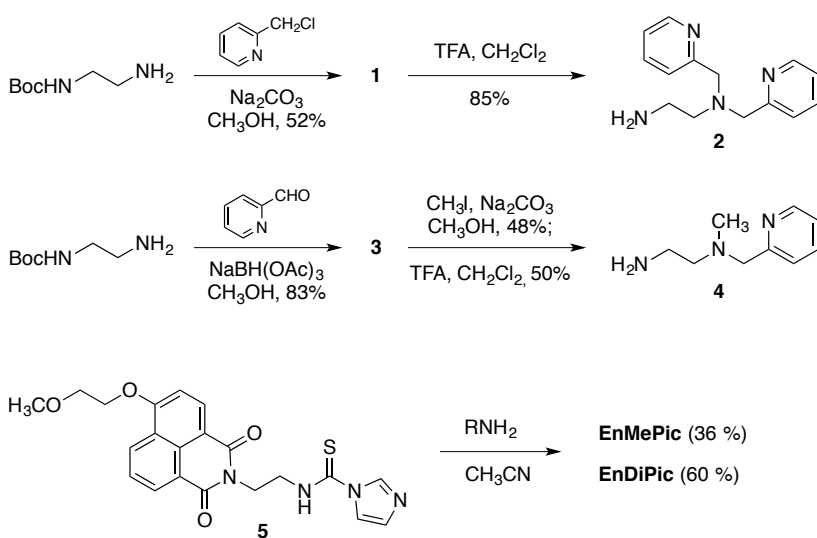


Figure 4. **EnMePic** and **EnDiPic**.



Scheme 1. Preparation of **EnMePic** and **EnDiPic**.

### *Metal Ion Titrations*

With the new probes in hand, metal ion titrations were performed in  $\text{CH}_3\text{OH}$  with  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Pb}^{2+}$ . Metal solutions were prepared from the corresponding metal chlorides, nitrates or perchlorates. The  $\text{CH}_3\text{OH}$  solutions (as opposed to aqueous) were chosen for initial evaluation to allow comparison with previous data and identify binding events too weak to be observed with  $\text{H}_2\text{O}$  as the competing solvent.

Metal ion response was observed for  $\text{Ag}^+$ , the  $d^{10}$  metals and  $\text{Pb}^{2+}$ , but not alkaline or alkaline earth cations.  $\text{Ag}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Pb}^{2+}$  titrations were then repeated in 9:1  $\text{H}_2\text{O}:\text{CH}_3\text{OH}$  (Tables 3, 4).<sup>11,16</sup> Focusing on the aqueous titrations, the magnitude of fluorescence response, represented by  $(I/I_0)_{\text{max}}$ , is generally lower than in  $\text{CH}_3\text{OH}$ , and the responses to  $\text{Ag}^+$  and  $\text{Pb}^{2+}$  are negated, as indicated by  $\log K_d$  values too high ( $> -1$ ) to be accurately determined. No

fluorescence enhancement for **EnMePic** or **EnDiPic** is observed at pH as low as 5.5.<sup>17</sup> The response of **EnMePic** in aqueous solution is diminished relative to **MePic** (e.g. there is no response to aqueous  $\text{Cd}^{2+}$ ). In contrast, the enhancements seen with **EnDiPic** are comparable or superior to those for **DiPic**, and are uniformly great enough ( $I/I_0 > 5$ ) to be potentially useful (Table 3). **EnMePic** and **EnDiPic** retain their high affinity for  $\text{Hg}^{2+}$  in 9:1  $\text{H}_2\text{O}:\text{CH}_3\text{OH}$ , but **EnDiPic** now has pronounced affinity for  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  as well (Table 4), exceeding the affinity for  $\text{Hg}^{2+}$ . The  $\log K_d$  values determined from the titrations are at the limit of measurement for the current reporting fluorophore, which is not bright enough to allow data acquisition below  $\sim 1 \mu\text{M}$  without repeat measurement. As a result, we think that the actual affinities are greater than the determined values, although the relative affinities are likely correct. For reasons discussed below, we have not pursued lower concentration measurements. The stoichiometry of the  $\text{Hg}^{2+}$  and  $\text{Cd}^{2+}$  complexes are 1:1, as determined by Job plot and/or Hill coefficient.<sup>18,19</sup> While **EnMePic**• $\text{Zn}^{2+}$  is also a 1:1 complex, **EnDiPic**• $\text{Zn}^{2+}$  has 2:1 L:M stoichiometry.

Table 3. Values of  $(I/I_0)_{\max}$  and  $\phi_{\max}$  for titrations of **EnMePic** and **EnDiPic**.<sup>a</sup>

	$\text{Zn}^{2+}$	$\text{Cd}^{2+}$	$\text{Hg}^{2+}$	$\text{Ag}^+$	$\text{Pb}^{2+}$
<hr/>					
$\text{CH}_3\text{OH}^b$					
<b>EnMePic</b>	7.4 (0.22)	5.1 (0.15)	6.3 (0.19)	5.2 (0.16)	–
<b>EnDiPic</b>	7.4 (0.30)	3.2 (0.13)	9.0 (0.36)	2.4 (0.10)	1.6 (0.06)
<hr/>					

H<sub>2</sub>O<sup>c</sup>

<b>EnMePic</b>	1.9 (0.06)	— <sup>d</sup>	2.5 (0.08)	— <sup>d</sup>	— <sup>d</sup>
<b>EnDiPic</b>	11.2 (0.45)	5.3 (0.21)	7.4 (0.30)	— <sup>d</sup>	— <sup>d</sup>

<sup>a</sup>  $\phi_{\max} = \phi_{\text{initial}} \times (I/I_0)_{\max}$ .  $\phi_{\max}$  in parentheses.  $\phi_0(\text{EnDiPic}) = 0.04$ ;  $\phi_0(\text{EnMePic}) = 0.03$ .

<sup>b</sup> Titrations in CH<sub>3</sub>OH at 3.3  $\mu$ M chemosensor.

<sup>c</sup> Titrations in 9:1 H<sub>2</sub>O:CH<sub>3</sub>OH at 3.3  $\mu$ M chemosensor.

<sup>d</sup> No detectable fluorescence response.

Table 4. Apparent logK<sub>d</sub> (M) for titrations of **EnMePic** and **EnDiPic**.

	Zn <sup>2+</sup>	Cd <sup>2+</sup>	Hg <sup>2+</sup>	Ag <sup>+</sup>	Pb <sup>2+</sup>
CH <sub>3</sub> OH <sup>a</sup>					
<b>EnMePic</b>	-5.0	-6.9	-5.9	-5.6	— <sup>c</sup>
<b>EnDiPic</b>	-7.1	-7.0	-6.0	-5.7	-5.4
H <sub>2</sub> O <sup>b</sup>					
<b>EnMePic</b>	-2.4	— <sup>c</sup>	-5.9	— <sup>c</sup>	— <sup>c</sup>
<b>EnDiPic</b>	-6.9	-7.2	-6.1	— <sup>c</sup>	— <sup>c</sup>

<sup>a</sup> Titrations in CH<sub>3</sub>OH at 3.3  $\mu$ M chemosensor.

<sup>b</sup> Titrations in 9:1 H<sub>2</sub>O:CH<sub>3</sub>OH at 3.3  $\mu$ M chemosensor.

<sup>c</sup> No detectable fluorescence response.

There is not a clear correlation between metal ion affinity and the magnitude of fluorescence response. This is consistent with a complexation model in which the affinity is determined by the ethylenediamine fragment and the fluorescence

response reflects the details of a second intramolecular equilibrium involving coordination of the thiourea (Figure 5). We have previously observed that thioureas have a low intrinsic affinity for metal ions, allowing us to exclude direct thiourea coordination as a significant process.<sup>6</sup>

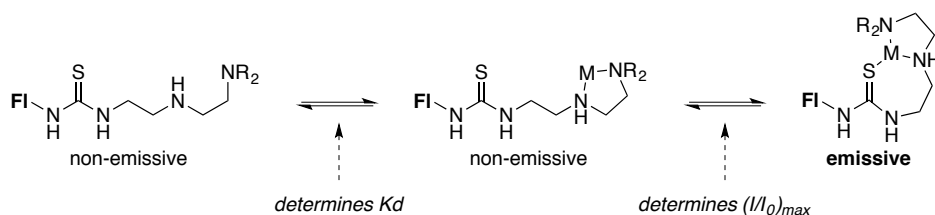


Figure 5. Proposed model for metal ion coordination. (FI = fluorophore; M = metal cation.)

The affinities and intensity of fluorescence response of **EnDiPic** would appear to set the stage for applied measurements. As it turns out, although the compounds are entirely stable on the timescale of our titrations,<sup>20</sup> we find that **EnMePic** and **EnDiPic** degrade over the course of a day in solution and 1-2 weeks as isolated solids. The degradation leads to the formation of complex mixtures of polar material, and appears to involve intramolecular cyclization coupled with dealkylation to form cyclic guanidine species. This conclusion is based on the high polarity and the MS analysis of crude degraded material; we have not yet been able to isolate purified degradation products.<sup>21</sup>

### Cellular Assays

We are unable to deploy **EnDiPic** in cellular assays due to its instability on the assay timescale. However, we have evaluated **DiPic** as a probe for  $\text{Hg}^{2+}$  in living human cells,  $\text{Hg}^{2+}$  being a logical starting point based on the high affinity and selectivity of **DiPic** for this ion. We found that **DiPic** is sufficiently membrane permeant to enter HeLa cells, and that it allows visualization of the presence of  $\text{Hg}^{2+}$  in a concentration dependent manner.<sup>11</sup> HeLa cells were incubated with **DiPic** (20  $\mu\text{M}$ ) overnight in standard DMEM growth media, followed by treatment with  $\text{HgCl}_2$  (200  $\mu\text{M}$ ) for 10 minutes, followed by fluorescence imaging. In the absence of either **DiPic** or  $\text{Hg}^{2+}$  ions, no detectable fluorescence was observed, similar to that of control cells treated with DMSO only (Figure 6a-c). However, upon treatment with both compound **DiPic** and  $\text{HgCl}_2$ , a significant increase in fluorescence appeared throughout the cells, but predominantly in the cytoplasm (Figure 6d). This demonstrates that the sensor **DiPic** is capable of detecting  $\text{Hg}^{2+}$  in live human cells. Moreover, **DiPic** successfully detected  $\text{Hg}^{2+}$  ions at lower concentrations of 50 and 100  $\mu\text{M}$  and the fluorescence intensity showed  $\text{Hg}^{2+}$  concentration dependence.<sup>11</sup>

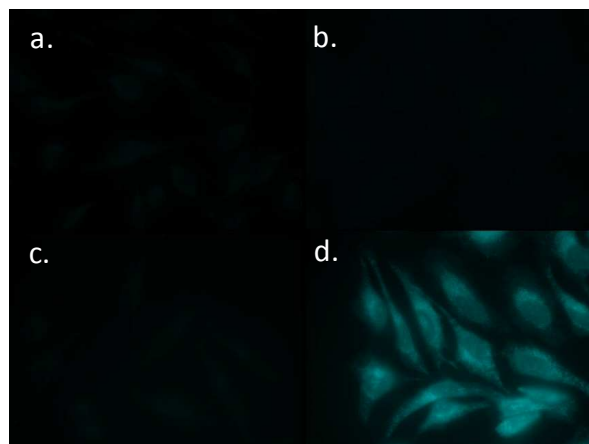


Figure 6. Fluorescence images of HeLa cells treated with **DiPic** and HgCl<sub>2</sub>. (a) HeLa cells treated with a DMSO control overnight at 37 °C. (b) Cells treated with a DMSO control followed by HgCl<sub>2</sub> (200 μM) for 10 min at 37 °C. (c) Cells incubated with **DiPic** (20 μM) overnight at 37 °C. (d) Cells treated with **DiPic** (20 μM) followed by HgCl<sub>2</sub> (200 μM) for 10 min at 37 °C. Imaging was performed on a Zeiss Axio Observer inverted microscope using a 63× objective and excitation and emission wavelengths of 365 nm and 445/50 nm, respectively.

While the practical value of imaging Hg<sup>2+</sup> in biological systems may be limited,<sup>4</sup> these data validate the use of PET-based thiourea probes in live cells, providing a solid precedent for the design and evaluation of future thiourea probes for relevant trace thiophilic ions, e.g., Fe<sup>2+</sup> or Cr<sup>3+</sup>.<sup>22</sup>

#### *Next Generation Thiourea Probes*

Two important issues must be addressed in the next generation of thiourea probes: enhancement of stability, and brightness of the reporting fluorophore. The need to improve stability is self-evident; we expect that, as observed with

first generation fluorophores,<sup>6</sup> *N*-methylation of the thiourea will improve stability. The use of more absorptive fluorophores will allow lower concentration measurements.

## Conclusions

We have shown that thiourea-naphthalimide conjugates with extended binding domains function as metal-responsive fluorescent chemosensors in aqueous media. They are capable of providing a useful ( $I/I_0 > 5$ ) turn-on response to  $Zn^{2+}$ ,  $Cd^{2+}$  and  $Hg^{2+}$  with at least high-nM sensitivity. In parallel, a previously reported thiourea has been shown to be a viable optical imaging agent for  $Hg^{2+}$  in live cells. Sufficient structural understanding has been attained to allow design of the next generation of improved thiourea-based fluorescent chemosensors, which should exhibit enhanced stability and improved measurement sensitivity, and be suitable for environmental and further cellular applications.

## Experimental Section

### *General Notes*

Imidazolyl thiourea **5**, **DiPic** and **MePic** were prepared as previously described.<sup>6</sup> All other reagents were used as received. Synthetic procedures were carried out under an inert atmosphere, in dry solvent, using standard Schlenk techniques, unless otherwise noted. Flash chromatographic purification was performed using silica gel Merck 60 (particle size 0.040–0.063 mm) packed in glass columns; eluting solvent for each purification was determined by thin layer chromatography



(TLC). Analytical thin-layer chromatography was performed using Merck TLC silica gel 60 F254 or Macherey–Nagel POLYGRAM ALOX N/UV254.

$^1\text{H}$  NMR chemical shifts are reported in parts per million (ppm) relative to the solvent residual peak ( $\text{CDCl}_3$ , 7.26 ppm). Multiplicities are given as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), and the coupling constants,  $J$ , are given in Hz.  $^{13}\text{C}$  NMR chemical shifts are reported relative to the solvent residual peak ( $\text{CDCl}_3$ , 77.0 ppm). HRMS data were acquired on Bruker maXis UHPLC-HR-MS QTOF instrument with an ESI source. All synthetic products were non-crystalline (oils or sticky solids), precluding melting point determination. Fluorescence measurements were carried out in spectroscopic grade  $\text{CH}_3\text{OH}$  using 450W Xenon lamp excitation with 1 nm excitation and 1 nm emission slit widths. Emission spectra were obtained by exciting at the longest-wavelength absorption maxima. For extinction coefficient determination, four independent solutions of different concentration were prepared, with absorption between 0.04-0.10 AU. The value of  $\epsilon$  was calculated by linear least-squares fitting of plots of  $A$  vs. concentration. All fits gave  $R^2$  values of  $\geq 0.98$ . Quantum yields for **EnMePic** and **EnDiPic** were determined by standard methods,<sup>11,23</sup> using anthracene ( $\phi = 0.30$ ) in  $\text{CH}_3\text{OH}$ .<sup>24</sup> The samples were diluted to optical transparency ( $A \leq 0.05$ ), and the integrated emission intensity was compared to an iso-absorptive solution of the standards in degassed solvent. Metal ion titrations were performed as previously described, using metal solutions prepared with spectroscopy grade  $\text{CH}_3\text{OH}$  or unbuffered,

purified H<sub>2</sub>O.<sup>6</sup>

### **Synthetic Procedures**

#### ***N,N*-di-(2-picolyl)-*N'*-Boc-ethylenediamine (1):**

2-Chloromethylpyridine hydrochloride (451 mg, 2.75 mmol) and Na<sub>2</sub>CO<sub>3</sub> (530 mg, 5.00 mmol) were added to a solution of *N*-Boc-1,2-diaminoethane (200 mg, 1.25 mmol) in methanol (20 mL), and the reaction mixture was heated to reflux for 48 hours. A 2N solution of NaOH (20 mL) was added and the product was extracted with dichloromethane. The organic phase was washed with brine, dried over MgSO<sub>4</sub> and concentrated under vacuum. The crude product was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub>; eluent: hexanes/CH<sub>2</sub>Cl<sub>2</sub>, 1:1 → CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 99:1) to give the product as a brown-yellow oil (220 mg, 52%). Spectroscopic data were consistent with those previously reported.<sup>13</sup>

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.55 (ddd, 2H, *J* = 4.9, *J* = 1.8, *J* = 0.9), 7.64 (td, 2H, *J* = 7.6, *J* = 1.8), 7.43 (d, 2H, *J* = 7.8), 7.16 (ddd, 2H, *J* = 7.5, *J* = 4.9, *J* = 1.2), 5.80 (s, 1H), 3.89 (s, 4H), 3.25-3.22 (m, 2H), 2.75-2.72 (m, 2H), 1.44 (s, 9H).

<sup>13</sup>C NMR (δ ppm, 125 MHz, CDCl<sub>3</sub>): 159.2 (2), 156.2, 149.1 (2), 136.5 (2), 123.2 (2), 122.1 (2), 78.8, 60.2 (2), 53.5, 38.5, 28.5 (3). HRMS-ESI: Calculated for C<sub>19</sub>H<sub>27</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup> 343.21333, found 343.21285, calculated for C<sub>19</sub>H<sub>26</sub>N<sub>4</sub>NaO<sub>2</sub> [M+Na]<sup>+</sup> 365.19510, found 365.19480. R<sub>f</sub> (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 99/1): 0.2

#### ***N,N*-di-(2-picolyl)ethylenediamine (2):**

**1** (80 mg, 0.23 mmol) was cooled to 0 °C in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) and trifluoroacetic acid (660 mg, 5.85 mmol) was added. The mixture was allowed to warm to room

temperature and stirred for 2 hours. 2N NaOH<sub>(aq)</sub> was added and the aqueous phase extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic fractions were dried over MgSO<sub>4</sub> and concentrated. **2** was obtained without purification as a yellow oil (48 mg, 85%). Spectroscopic data were consistent with those previously reported.<sup>13</sup>

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.54 (dd, 2H, *J* = 4.9, *J* = 1.3), 7.65 (td, 2H, *J* = 7.7, *J* = 1.8), 7.48 (d, 2H, *J* = 7.8), 7.15 (ddd, 2H, *J* = 7.4, *J* = 4.9, *J* = 1.2), 3.86 (s, 4H), 2.82 (t, 2H, *J* = 6.0), 2.69 (t, 2H, *J* = 6.0). <sup>13</sup>C NMR (δ ppm, 100 MHz, CDCl<sub>3</sub>): 159.5 (2), 149.1 (2), 136.5 (2), 123.0 (2), 122.1 (2), 60.5 (2), 56.3, 39.3. HRMS-ESI: Calculated for C<sub>14</sub>H<sub>19</sub>N<sub>4</sub> [M+H]<sup>+</sup> 243.16020, found 243.16045.

**4-(2-Methoxyethoxy)-*N*-(ethyl-1-(2-(bis(pyridin-2-ylmethyl) amino)ethyl) thiourea)naphthalimide (EnDiPic):**

**5** (53 mg, 0.12 mmol); acetonitrile (5 mL) and **2** (30 mg, 0.12 mmol) were combined in CH<sub>3</sub>CN (5 mL) and stirred for 2 hour at reflux. After removal of the solvent, the crude product was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub> → CH<sub>2</sub>Cl<sub>2</sub>:MeOH 99:1) to give the product **EnDiPic** (45 mg, 60%) as a yellow solid.

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.64 (dd, 1H, *J* = 1.6, *J* = 8.4), 8.59 (dd, 1H, *J* = 1.2, *J* = 7.3), 8.57-8.56 (m, 2H), 8.54 (d, 1H, *J* = 8.3), 7.71 (dd, 1H, *J* = 7.3, *J* = 8.4), 7.59 (dt, 2H, *J* = 1.8, *J* = 7.6), 7.35-7.33 (m, 2H), 7.12-7.09 (m, 2H), 7.04 (d, 1H, *J* = 8.3), 4.50-4.48 (m, 2H), 4.45-4.43 (m, 2H), 3.96-3.93 (m, 4H), 3.85 (s, 4H), 3.53 (s, 3H), 2.82 (t, 2H, *J* = 5.6). <sup>13</sup>C NMR (δ ppm, 100 MHz, CDCl<sub>3</sub>): 181.9, 165.4, 164.8, 160.6, 159.0 (2), 149.3 (2), 136.7 (2), 134.0, 132.1, 129.7, 129.5,

126.2, 123.8, 123.3 (2), 122.3 (2), 122.1, 114.9, 106.3, 70.8, 68.7, 59.9, 59.6, 52.3, 43.0, 39.2. HRMS-ESI: Calculated for  $C_{32}H_{34}N_6O_4S$   $[M+H]^+$  599.24350, found 599.24344; calculated for  $C_{32}H_{33}N_6NaO_4S$   $[M+Na]^+$  621.22545, found 621.22522.  $R_f$  ( $Al_2O_3$ ,  $CH_2Cl_2$ :MeOH 99.5:0.5): 0.25.

***N*-(2-picolyl)-*N'*-Boc-ethylenediamine (3):**

*N*-Boc-1,2-diaminoethane (627 mg, 3.92 mmol) was dissolved in methanol (15 mL) and 2-pyridine carboxaldehyde (350 mg, 3.27 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 3 hours, sodium triacetoxyborohydride (970 mg, 4.57 mmol) was added and the reaction mixture was stirred overnight. The solvent was removed under vacuum and the residue was treated with saturated  $Na_2CO_3$ . The product was extracted with  $CH_2Cl_2$ , and the organic phase was dried over  $MgSO_4$  and concentrated under vacuum. **3** was obtained as a brown oil (680 mg, 83%) in sufficient purity for use in the subsequent step. Spectroscopic data were consistent with those previously reported.<sup>14</sup>

$^1H$  NMR ( $\delta$  ppm, 400 MHz,  $CDCl_3$ ): 8.56 (dd, 1H,  $J = 5.0, 1.2$ ), 7.65 (td, 1H,  $J = 7.7, J = 1.8$ ), 7.29 (d, 1H,  $J = 7.8$  Hz), 7.18 (ddd, 1H,  $J = 7.5, J = 4.9, J = 1.1$ ), 5.17 (s, 1H), 3.93 (s, 2H), 3.39-3.36 (m, 2H), 2.82-2.80 (m, 2H), 1.44 (s, 9H).  $^{13}C$  NMR ( $\delta$  ppm, 125 MHz,  $CDCl_3$ ): 158.9, 156.2, 149.4, 136.6, 122.4, 122.2, 79.2, 54.5, 48.8, 40.2, 28.4 (3).  $R_f$  ( $Al_2O_3$ ,  $CH_2Cl_2$ :MeOH 99.5:0.5): 0.11

***N*-methyl-*N*-(2-picolyl)-*N'*-Boc-ethylenediamine (Boc-protected 4):**

Iodomethane (34 mg, 0.239 mmol) and sodium carbonate (85 mg, 0.80 mmol)

were added to a solution of **3** (50 mg, 0.20 mmol) in CH<sub>3</sub>OH (20 mL) and the reaction mixture was heated to reflux for 72 hours. The solvent was concentrated under vacuum and the crude product was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub>, eluent: CH<sub>2</sub>Cl<sub>2</sub>). The product was obtained as a light yellow oil (25 mg, 48%).

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.57-8.55 (m, 1H), 7.67 (td, 1H, *J* = 7.7, *J* = 1.8), 7.38 (d, 1H, *J* = 7.9), 7.18 (dd, 1H, *J* = 7.4, *J* = 3.0), 3.71 (s, 2H), 3.27-3.25 (m, 2H), 2.59-2.57 (m, 2H), 2.32 (s, 3H), 1.45 (s, 9H). <sup>13</sup>C NMR (δ ppm, 125 MHz, CDCl<sub>3</sub>): 159.0, 156.2, 149.3, 136.6, 123.2, 122.2, 79.2, 63.8, 56.5, 42.4, 38.2, 38.6 (3). HRMS-ESI: Calculated for C<sub>14</sub>H<sub>24</sub>N<sub>3</sub>O<sub>2</sub> [M+H]<sup>+</sup> 266.1863, found 266.1864. R<sub>f</sub> (aluminium oxide: CH<sub>2</sub>Cl<sub>2</sub>): 0.31

***N*-methyl-*N*-(2-picolyl)ethylenediamine (**4**):**

*N*-methyl-*N*-(2-picolyl)-*N*'-Boc-ethylenediamine (150 mg, 0.57 mmol) was cooled to 0 °C in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and trifluoroacetic acid (1.61 g, 14.15 mmol) was added. The mixture was allowed to warm to room temperature and stirred for 2 hours. 2N NaOH<sub>(aq)</sub> was added and the aqueous phase extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic fractions were dried over MgSO<sub>4</sub> and concentrated. **4** was obtained without purification as a yellow oil (48 mg, 50%) and used immediately in the next reaction.

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.57-8.55 (m, 1H), 7.68-7.64 (m, 1H), 7.38 (d, 1H, *J* = 7.9), 7.19-7.17 (m, 1H), 3.68 (s, 2H), 2.56-2.54 (m, 4H), 2.26 (s, 3H).

**4-(2-methoxyethoxy)-*N*-(ethyl-1-(2-methyl(2-picolyl)amino)ethyl) thiourea)**

**naphthalimide (EnMePic):**

**5** (103 mg, 0.24 mmol); acetonitrile (5 mL) and **4** (40 mg, 0.24 mmol) were combined in CH<sub>3</sub>CN (5 mL) and stirred for 2 hour at reflux. The solvent was removed and the crude product was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub> → CH<sub>2</sub>Cl<sub>2</sub>:MeOH 99:1) to give the product **EnMePic** as a yellow solid (45 mg, 36%).

<sup>1</sup>H NMR (δ ppm, 400 MHz, CDCl<sub>3</sub>): 8.66 – 8.55 (m, 3H), 8.53 (d, 1H, *J* = 8.3), 7.70 (dd, 1H, *J* = 7.3, *J* = 8.4), 7.62 (td, 1H, *J* = 1.8, *J* = 7.7), 7.34 (d, 1H, *J* = 7.8), 7.14 (dt, *J* = 2.9, *J* = 8.1), 7.05 (d, 1H, *J* = 8.3), 4.57-4.36 (m, 4H), 4.01-3.81 (m, 4H), 3.73 (s, 2H), 3.52 (s, 5H), 2.71 (s, 2H), 2.31 (s, 3H). <sup>13</sup>C NMR (δ ppm, 125 MHz, CDCl<sub>3</sub>): 182.0, 165.4, 164.7, 160.6 (2), 149.4, 136.8, 134.0, 132.1, 129.6, 129.6, 126.2, 123.7, 123.4, 122.4, 122.0, 114.8, 106.0, 70.8, 68.6, 63.3, 59.6, 55.4, 53.6, 42.2, 39.1. HRMS-ESI: Calculated for C<sub>27</sub>H<sub>32</sub>N<sub>5</sub>O<sub>4</sub>S [M+H]<sup>+</sup> 522.21695, found 522.21711. R<sub>f</sub> (Al<sub>2</sub>O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>:MeOH 99.5:0.5): 0.25.

**Cell culture.**

Experiments were performed using HeLa cells (ATCC) cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 2% penicillin/streptomycin (Mediatech) and maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere.

**Detection of Hg<sup>2+</sup> ions in mammalian cells.**

HeLa cells were passaged into an 8-well chamber slide at 10,000 cells/well and were grown overnight at 37 °C. The media was removed and the cells were treated with either a DMSO control (1% DMSO final concentration) or **DiPic** (20  $\mu$ M) in DMEM and were incubated overnight at 37 °C. The media was removed and the cells were washed 3  $\times$  with PBS (100  $\mu$ L). The cells were then treated with HgCl<sub>2</sub> at 0 or 200  $\mu$ M in PBS and were incubated for 10 min at 37 °C. The cells were imaged for fluorescence on a Zeiss Axio Observer inverted microscope using a 63 $\times$  objective and filter set 49 (excitation 365 nm, emission 445/50 nm).

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## **Supporting Information Available**

Supporting Information: Representative absorbance spectra, emission spectra, titration spectra; concentration-dependent Hg<sup>2+</sup> imaging in HeLa cells; copies of <sup>1</sup>H and <sup>13</sup>C NMR spectra; crystallographic data (CIF) for the ZnCl<sub>2</sub> complex of the truncated ligand. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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15. **EnMePic**:  $\varepsilon = 13,800 \text{ cm}^{-1}\text{M}^{-1}$ ;  $\phi = 0.03$ ;  $\lambda_{\text{max(abs)}} = 368 \text{ nm}$  (longest-wavelength maximum);  $\lambda_{\text{max(em)}} = 446 \text{ nm}$ . **EnDiPic**:  $\varepsilon = 14,500 \text{ cm}^{-1}\text{M}^{-1}$ ;  $\phi = 0.04$ ;  $\lambda_{\text{max(abs)}} = 368 \text{ nm}$  (longest-wavelength maximum);  $\lambda_{\text{max(em)}} = 446 \text{ nm}$ .

16. Binding constants were determined by non-linear least-squares fitting of plots of emission intensity vs log[M] using the program Prism3 (Graphpad, Inc., San Diego, CA).

17. The addition of HCl to a solutions of **EnMePic** or **EnDiPic** do not produce a

change in fluorescence until ~ pH 5. At this point, the protonated picolylamine begins to serve as an alternate PET acceptor and fluorescence begins to increase. For precedent, see: de Silva, S. A.; Zavaleta, A.; Baron, D. E.; Allam, O.; Isidor, E. V.; Kashimura, N.; Percarpio, J. M. *Tetrahedron Lett.* **1997**, *38*, 2237-2240.

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20. We have found that **EnMePic** and **EnDiPic** can be recovered untransformed following exposure to  $\text{Zn}^{2+}$  and  $\text{Hg}^{2+}$  for several hours in  $\text{CH}_3\text{OH}$ . This excludes the possibility that fluorescence enhancements are the result of desulfurization of the thiourea. The addition of chelating agents (EDTA or dipicolyl amine) reverses the observed fluorescence enhancements.

21. There is a precedent for metal-ion induced cyclization of *N*-( $\beta$ -aminoethyl) *N'*-acylthioureas to form cyclic guanidines in the context of chemodosimeters, in which a  $\beta$ -amino NH serves as the nucleophile. (See refs. 1a, d.) We had anticipated that the absence of a free  $\beta$ -NH would suppress such cyclization.

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